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N_2 gas plasma inactivates influenza virus mediated by oxidative stress

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1. ABSTRACT

Here we show that N_2 gas plasma, produced by applying a short high-voltage pulse using a static induction (SI) thyristor power supply inactivates influenza virus. N_2 gas plasma treatment of influenza A and B viruses induced the degradation of viral proteins, including nucleoprotein, hemagglutinin, and neuraminidase. The injury of viral RNA genome and the inactivation of hemagglutination were also observed after N_2 gas plasma treatment. These changes were possibly due to changes in the viral envelope, because modification of the lipid content was also suggested by Fourier-transformed infrared spectroscopy. At least three major mechanisms of action [heat, UV-A, and oxidative stress (i.e. hydrogen peroxide-like molecules)] were found in this system. Among them, oxidative stress appeared to be the main factor in the inactivation of influenza virus. In addition, there was an increase in the nitrotyrosine content of viral proteins, suggesting that oxidative stress produced by N_2 gas plasma generation oxidized proteins. As a result, oxidation may be the most important factor in the inactivation, degradation, and modification of influenza virus by N_2 gas plasma.

2. INTRODUCTION

The influenza virus is about 80 to 120 nm in diameter, and has a lipid membrane called an envelope (1, 2). The envelope contains a protein called hemagglutinin (HA), which is required for adsorption of the virus into a cell, and neuraminidase (NA), which is required for release of viruses from the cell. In addition, RNA entangled with a protein called nucleoprotein (NP) exists in the central part of the virus. The lipid bilayer of envelope of the influenza virus is supported by a matrix protein (M1) and spanned by an ion channel (M2). In a previous study, we reported biochemical changes in NP of influenza virus after N_2 gas plasma treatment produced by a gas plasma apparatus, BLP-TESNo.1 (NGK Insulators, Ltd, Nagoya, Japan) (3). Similarly, we showed inactivation of spore forming and non-forming bacteria, and endotoxins (4, 5). The N_2 gas plasma device produces an N_2 gas plasma by means of a short high-voltage pulse generated using a static induction (SI) thyristor power supply. Because influenza viruses infect respiratory tracts and cause influenza especially in winter, inactivation of influenza virus is important for preventing this disease. Thus, N_2 gas plasma could be used for sterilization or disinfection, or as an antiseptic.

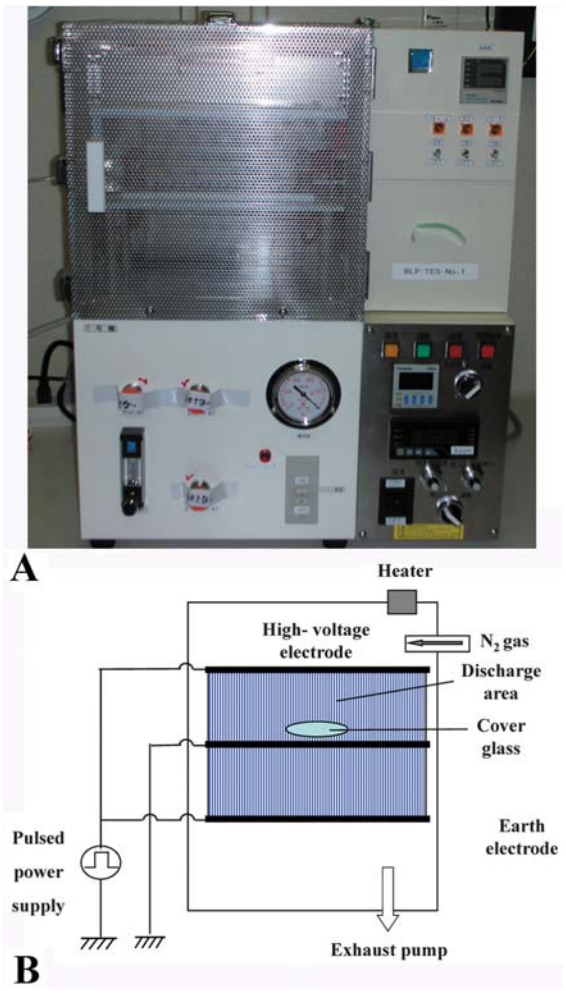

Figure 1. Experimental setup for N_2 gas plasma production. (A) Photo of the N_2 gas plasma instrument (BLP-TESNo.1). (B) Schematic of the N_2 gas plasma instrument. The distance between the high voltage electrode and the earth electrode was 50 mm. The flow rate of N_2 gas was 10 L/min. During N_2 gas plasma generation, the sample box was kept at a pressure of 0.5 atm. A static induction (SI) thyrister was used for the pulsed power supply.

The gas plasma is generated by removing electrons from the gas, which produces a highly excited mixture of charged nuclei and free electrons (6, 7). During the process, several other factors such as heat, ultraviolet (UV) radiation, and oxidative stress are generated. Our N_2 gas plasma apparatus (BLP-TESNo.1), by which influenza virus was inactivated, may produce elevated temperatures, UV rays, and oxidative stress; however, it remains unclear which is the major mechanism of action. In this study, therefore, we investigated which factors constitute the main mechanism of disinfection by N_2 gas plasma.

3. MATERIALS AND METHODS

3.1. N_2 gas plasma measurement and virus treatment

BLP-TESNo.1 produced the N_2 gas plasma by means of a fast high-voltage pulse applied using a SI thyristor power supply (Figure 1A). A cathode electrode (earth electrode) was placed between the anode electrodes (high voltage electrode) (Figure 1B). A 20-μL aliquot of influenza virus-infected allantoic fluid from an infected embryonated egg was dropped onto a cover glass, air-dried, and then placed on the grid of electrodes. The procedure for generating the N_2 gas plasma was as follows. First, the chamber box containing the sample was decompressed and degassed, and then N_2 gas (99.9995%, Okano, Okinawa, Japan) was introduced. The pressure in the box was maintained at about 0.5 atmospheres during the discharge at 1.5 kpps (kilo pulse per second). The N_2 gas plasma-treated samples and untreated samples on a cover glass were subsequently recovered in 20 μL of pure water (Otsuka Pharmaceuticals Co., Tokyo, Japan).

3.2. Allantoic fluid

Influenza virus-infected allantoic fluid was prepared from chicken embryonated eggs injected with influenza virus as described previously (8).

3.3. Purified influenza A and B virus

Allantoic fluids of embryonated eggs infected with either influenza A virus H1N1 (A/Taiwan/1/86), H3N2 (A/Panama/2007/99) or influenza B virus (B/Tokio/53/99) were collected and subjected to fractionation using a sucrose density gradient generated by ultracentrifugation. The fractions obtained were checked by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), followed by Coomassie Brilliant Blue (CBB) staining, which showed that the purity of the influenza A and B viruses was over 99%.

3.4. Chemical indicators

STERRAD indicator strips (Johnson & Johnson, New Brunswick, NJ) were used as indicators of oxidative stress (hydrogen peroxide-like molecules).

3.5. Treatment with reagents or heating

For heat treatment, influenza virus-infected allantoic fluid was incubated at temperatures ranging from 35 to 65 ºC for 5 min using a block incubator (BI-516S; ASTEC, Fukuoka, Japan). For UV treatment, influenza virus-infected allantoic fluid was dried onto a cover glass and exposed to UV-A and UV-C from a handheld UV lamp (UVGL-58, UVP) for 5 min. For oxidative stress treatment, influenza virus-infected allantoic fluid was incubated in the presence of hydrogen peroxide (0-3%) (WAKO, Osaka, Japan), peroxynitrite (0-4.5 mM) (Dojindo, Kumamoto, Japan), and 3-(4-Morpholinyl) sydnonimine hydrochloride (SIN-1) (0-1mM) (Dojindo, Kumamoto, Japan) for 5 min. $\cdot O_2^-$ radical ions were generated by a xanthine-oxidase system (Dojindo, Kumamoto, Japan). In all cases, the treated and untreated influenza virus was injected into 11-day-old chicken embryonated eggs, which were then incubated for 48 h at 37ºC.

3.6. Fourier-transformed infrared (FT-IR)

Attenuated total reflection (ATR) FT–IR spectra (1800–800 cm^{-1}) were collected at 4 cm^{-1} resolution by using an FT–IR spectrophotometer (Nicolet 6700; Thermo Fisher Scientific K.K., Waltham, MA) with ATR equipment containing a ZnSe ATR crystal, an

electronically temperature controlled (ETC)–Ever-Glo IR source, a deuterated lanthanum triglycine sulfate (DLATGS) detector, and a KBr beam-splitter under purging with a continuous flow of dried air (Air Tech Japan Ltd., Tokyo, Japan). The wavenumbers of near 1655 cm^{-1} (Amide I of proteins), 1400-1300 cm^{-1} (CH_2 wagging vibration bands of lipids), and 1160-1000 cm^{-1} (C-O stretch of carbohydrates) peaks assigned to protein, liquid, and carbohydrate (9) were compared between N_2 gas plasma-treated and untreated samples.

3.7. Spectral analysis of emission

Emission from the N_2 gas plasma instrument in the spectral region 200-800 nm was collected via a multichannel spectrophotometer (S-2431, Soma Optics Ltd., Tokyo, Japan) attached to a fiber probe.

3.8. Gas analysis

The exhaust gas from the N_2 gas plasma apparatus during the experiment was analyzed by a conventional gas detector (Kitagawa Gas detector Tube System; Komyo Rikagaku Kogyo K.K., Kawasaki, Japan).

3.9. Temperature measurement

The ambient temperature in the N_2 gas plasma instrument box was measured by a fiber optic thermometer (FT1420A, Takaoka Electric MFG. Co. Ltd., Tokyo, Japan).

3.10. Hemagglutination assay

Samples were serially diluted two-fold in 50 µl of phosphate buffered saline (PBS) in V-shaped wells in an assay plate, and an equal volume of 1% chicken erythrocytes in suspension was added. The mixture was then incubated at room temperature for 1 h. The agglutination pattern was read and the hemagglutination titer was defined as the reciprocal of the last dilution of the sample that showed hemagglutination.

3.11. Enzyme linked immunosorbent assay (ELISA) for influenza virus

A Serion ELISA antigen Influenza B virus test (Institut Virion/Serion GmbH, Würzburg, Germany) was used for quantification of influenza B virus NP.

3.12. Real-time polymerase chain reaction (rPCR) for influenza virus

Viral genomic RNA was extracted from untreated and N_2 gas plasma-treated allantoic fluid infected with influenza A virus (A/PR/8/34) by using a QIAamp Viral RNA mini kit (Qiagen, Hilden, Germany). The RNA was transcribed with a PrimeScriptII 1st strand cDNA Synthesis kit (Takara Bio Inc., Otsu, Japan) and the following temperature regime: 65ºC for 5 min, 4ºC for 5 min, 42ºC for 60 min. The resultant cDNAs were subjected to rPCR using SYBR Premix Ex TaqII (Tli RNase H Plus) (Takara Bio Inc.) and the following primers: MA1-F: 5'-GGG ATC CAA ATA ACA TGG ACA AAG-3'; MA1-R: 5'-GCA CCA GCA GAA TAA CTG AGT GAG A-3'; HA-F: 5'-CGC ATC AAT GCA TGA GTG TAA -3'; HA-R: 5'-CAA TTT GGC ACT CCT GAC GTA -3'; NA-F: 5'-AGG CAA GAT GTT GTG GCA ATG A-3'; NA-R: 5'-AAC CCA GAA GCA CGG CCT TAT AC-3'; ; NS-F: 5'-TGT ACC TGC GTC GCG TTA CCT A-3'; NS-R: 5'-ATG ATC GCC TGG TCC ATT CTG-3'. The temperature cycling conditions used for the rPCR were: 95ºC for 30 sec, followed by 40 cycles of 95ºC for 5 sec, and 60ºC for 1 min.

3.13. Scanning electron microscopy (SEM)

The influenza virus-infected allantoic fluids were air-dried on a cover glass, treated by N_2 gas plasma, and then fixed with 2% glutaraldehyde/0.1 M phosphate buffer (pH 7.4) overnight at 4ºC. The cover glasses were subsequently treated with 2% osmium tetraoxide at 4ºC for 3 h. Samples were dehydrated through a graded ethanol series (50%–100% ethanol) at room temperature. Finally, the cover glass was subjected to critical point drying and evaporation coating by osmium plasma ions. SEM was performed using a JSM-6320F (JEOL Ltd., Tokyo, Japan) instrument at a magnification of x100,000.

3.14. Immunochromatography

An immunochromatography test assembly developed using anti-influenza virus and anti-immunoglobulin (Ig) antibodies (ESPRINE® Influenza A&B-N, Fujirebio Inc., Tokyo, Japan) was used. To capture influenza virus NP, anti-influenza A and B NP antibodies were immobilized onto a nitrocellulose membrane for test lines (lines A and B, respectively). A reference line was also prepared by immobilizing anti-Ig antibody onto a nitrocellulose membrane to capture Ig. A conjugated pad containing anti-influenza virus NPs used for the test line was labeled with colloidal gold, impregnated onto glass fibers, dried, and then placed between the test lines and the sample dropping region. The nitrocellulose membrane was assembled with a glass fiber pad on a plastic sheet within a plastic case. This assembled kit was stored in a bag with desiccant at 4°C until required. The kit was then used to quantify the amount of influenza virus NPs.

3.15. Western blotting

Samples were solubilized in an equal volume of 2× sodium dodecyl sulfate (SDS) gel-loading buffer [90 mM Tris–HCl (pH 6.8), 10% mercaptoethanol, 2% SDS, 0.02% bromophenol blue, and 20% glycerol], boiled for 5 min, and then separated on an SDS–8% polyacrylamide gel electrophoresis (PAGE) gel, before being electrically transferred onto a polyvinylidene fluoride (PVDF) membrane (Hybond-P; Amersham-Pharmacia Biotech, Piscataway, NJ) for 60 min at 15 V. Blots were treated with 5% skim milk for 1 h at room temperature, and then incubated with a polyclonal anti-human influenza A virus NP antibody (IMG-5134A, IMGENEX Corp., San Diego, CA), anti-NA antibody (IMG-6460A, IMGENEX Corp.), or anti-nitrotyrosine antibody (MAB5404, Millipore, Billerica, MA) in PBS containing 0.1% Tween 20 (PBS-T) and 0.5% skim milk for 1 h at room temperature. After three washes with PBS-T, the membrane was incubated in horseradish peroxidase (HRP)-conjugated anti-mouse IgG or anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc., West Grove, PA) in PBS-T and 0.5% skim milk for 1

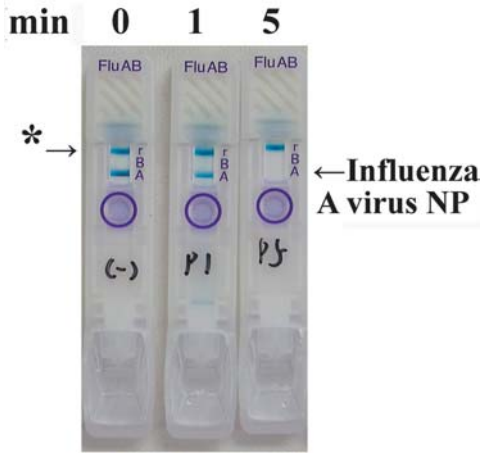

Figure 2. Inactivation of influenza A virus by N_2 gas plasma within 5 min. Spots of allantoic fluid ($3x10^{14}$ $TCID_{50}$/ml) infected with influenza A virus (A/PR/8/34) were air-dried on a cover glass and treated with N_2 gas plasma (1.5 kpps) for 0, 1, or 5 min) using BLP-TESNo.1, and injected into embryonated eggs. After incubation of the eggs for 48 h, the presence of influenza virus was analyzed by immunochromatography for influenza virus nucleoprotein (NP) (ESPRINE Influenza A&B-N, Fujirebio Inc.) N_2 gas plasma treatment (1.5 kpps) effectively inactivated influenza virus within 5 min. Arrow and asterisk indicate the lines for influenza A virus NP and the reference, respectively.

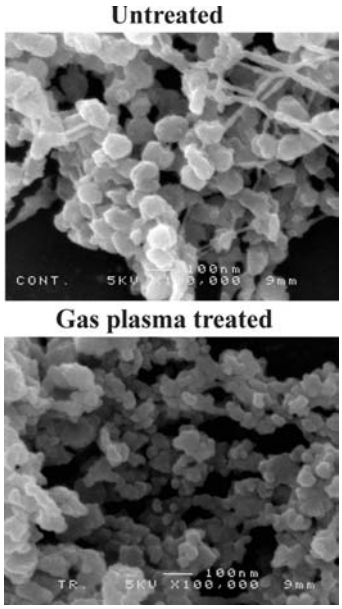

Figure 3. Change in morphology of influenza virus treated with N_2 gas plasma (x100,000). Influenza A virus (A/PR/8/34) in allantoic fluid was air-dried on a cover glass and treated for 5 min with an N_2 gas plasma (1.5 kpps) generated using BLP-TESNo.1. The morphology of the virions was observed by scanning electron microscopy (SEM) at 5 kV at a magnification of x100,000. Untreated influenza A virus showed a round shape with fibrous connections, whereas N_2 gas plasma-treated influenza A virus showed a shrunken form without fibers.

h at room temperature. After three washes with PBS-T, the probed proteins were detected using an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech).

3.16. Measurement of $TCID_{50}$ (tissue culture infectious dose 50)

Viral titers were determined by performing 10-fold serial dilutions of samples in 96-well plates containing Madin-Darby Canine Kidney (MDCK) cells with 8 replicates for each sample. Before infection, cells were washed with PBS. Infected cells were incubated at 37°C with 5% CO_2 and fresh trypsin was added (final concentration, 0.03%). Viral titers were read as $TCID_{50}$ calculated by the method of Reed & Muench (10).

4. RESULTS

First, the infectivity of influenza virus treated with N_2 gas plasma was investigated using chicken embryonated eggs. Influenza virus (A/PR/8/34)-infected allantoic fluid dried on a cover glass was subjected to treatment with N_2 gas plasma (1.5 kpps; 0, 1, 5 min) (Figure 2). Samples were collected with pure water and injected into embryonated eggs, which were then incubated for 48 h. The allantoic fluids were collected and subjected to immunochromatography for influenza virus NP (ESPLINE® Influenza A&B-N, FUJIREBIO Inc.). NP was not detected in fluid from embryonated eggs that had been treated with N_2 gas plasma for 5 min (Figure 2). Specifically, a band corresponding to NP in test line A was detected at 0 and 1 min, but was absent at 5 min. A band was detected in the reference line at all time points (0, 1, 5min) indicating that the immunochromatography was working as anticipated. On the basis of this result, we concluded that influenza virus was inactivated within 5 min of N_2 gas plasma treatment.

Next, the morphology of influenza virus treated with N_2 gas plasma was observed by SEM (Figure 3). At a magnification of x100,000, SEM showed that N_2 gas plasma treatment (1.5 kpps, 5 min) disrupted fibers connecting influenza virus particles in the allantoic fluid. Moreover, the N_2 gas plasma-treated influenza virus particles (diameter ~100 nm) displayed a shrunken appearance. In addition, fused influenza virus particles were also observed in the treated samples, suggesting that the N_2 gas plasma modified the viral envelope.

The effect of the N_2 gas plasma on biomolecules in the culture medium of influenza virus-infected cells was analyzed by FT-IR (Figure 4). N_2 gas plasma-treated influenza virus was solubilized in pure water, and then subjected to FT-IR analysis. Comparison of the absorbance between treated and untreated influenza virus indicated that a new peak in the region assigned to lipids (Figure 4, arrow) appeared after 7.5 min of treatment. These findings are consistent with the SEM observation of shrunken or fused viruses after N_2 gas plasma treatment, and support modification of the viral surface (i.e., envelope) containing lipid.

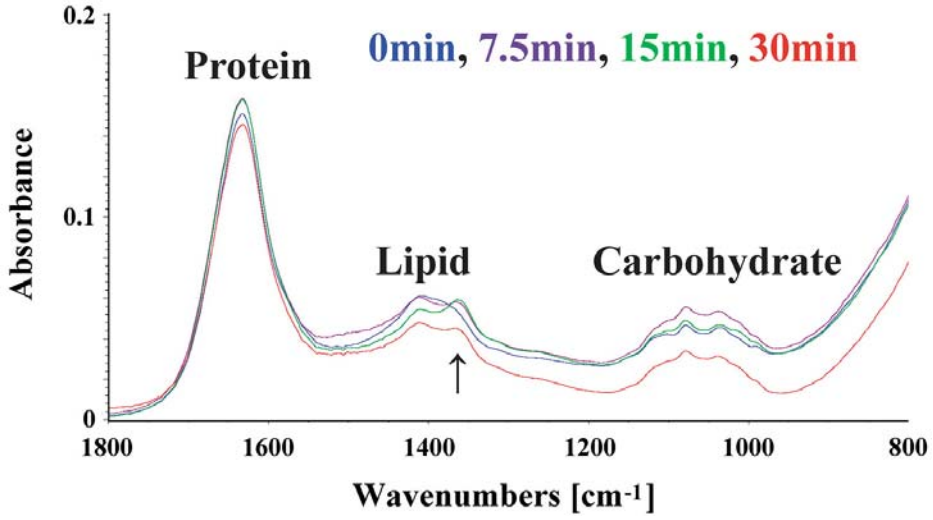

Figure 4. Modification of influenza A virus lipid by N_2 gas plasma treatment.Influenza A virus (A/PR/8/34) ($3X10^{14}$ $TCID_{50}$/ml) derived from the cell culture medium of Madin-Darby canine kidney (MDCK) cells that had been incubated for 24 h after infection was treated with N_2 gas plasma at 1.5 kpps for 0 min (blue), 7.5 min (purple), 15 min (green), or 30 min (red) Samples were collected in distilled water and subjected to attenuated total reflection (ATR) Fourier-transformed infrared (FT-IR) analysis. The monitored wavenumbers ranged from 1800 to 800 cm^{-1}, which includes spectral regions related to proteins, lipids, and carbohydrates, whose peaks are indicated. The peak assigned to lipid was increased after N_2 gas plasma treatment (arrow).

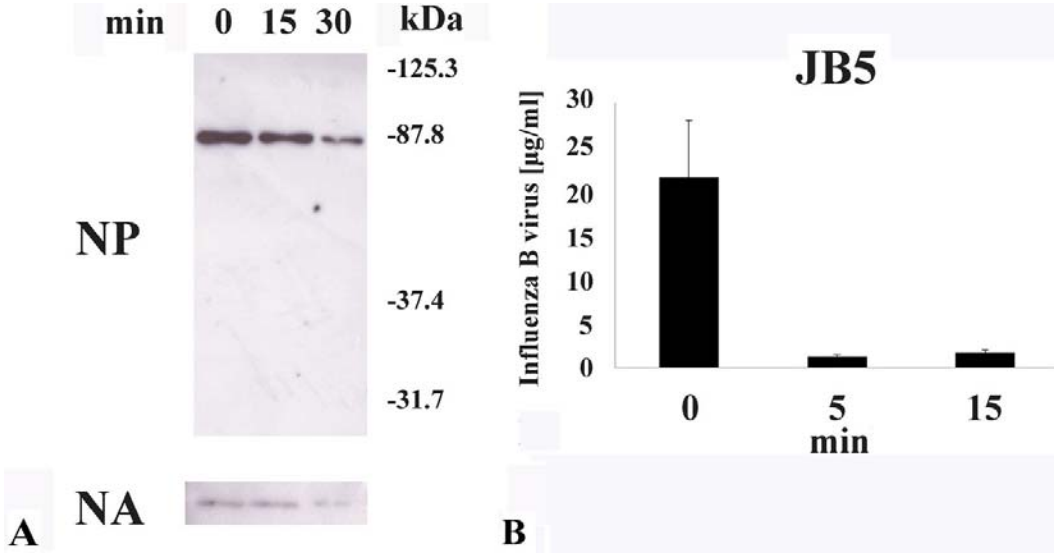

Figure 5. Degradation of viral proteins in influenza A virus by N_2 gas plasma.(A) Influenza A virus (A/PR/8/34) in allantoic fluid of influenza virus-infected embryonated egg ($3X10^{14}$ $TCID_{50}$/ml) before and after treatment with N_2 gas plasma. Allantoic fluid (20 μl) was dried on a cover glass and treated with N_2 gas plasma (1.5 kpps, 15 min) Degradation of influenza A virus nucleoprotein (NP) and neuraminidase (NA) was observed by western blotting after N_2 gas plasma treatment for 0, 15, or 30 min. The molecular weight marker is shown in the right side. (B) Quantity of influenza B viruses (JB5: B/Johannesburg/5/99) derived from infected allantoic fluid before and after treatment with N_2 gas plasma. Allantoic fluid (20 μl) was dried on a cover glass and treated with N_2 gas plasma at 1.5 kpps for 0 and 5 min. The quantity of NP of influenza B virus was measured by an enzyme-linked immunosorbent assay against NP.

To investigate the effect of N_2 gas plasma on viral proteins including HA, NA, and NP, western blotting, ELISA and hemagglutination assay were performed. Western blotting showed that the amount of NP and NA had decreased after 15 and 30 min as compared with 0 min, suggesting that these viral proteins were degraded by the N_2 gas plasma (Figure 5A). Similarly, ELISA using anti-influenza B virus NP antibody showed degradation of NP in influenza B virus treated with N_2 gas plasma (Figure 5B). Within 5 min, the concentration of influenza B virus

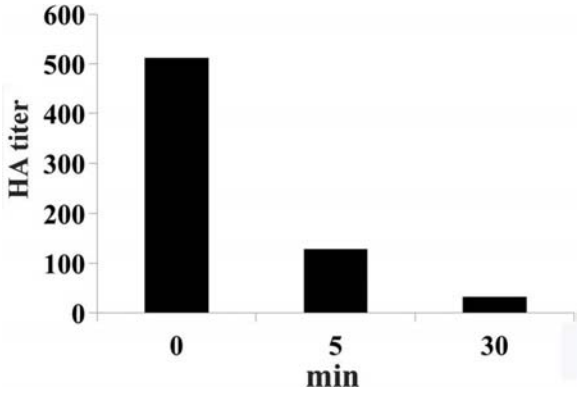

Figure 6. Inhibition of the hemagglutination activity of influenza virus HA.Influenza A virus (A/PR/8/34) derived from infected allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was treated with N_2 gas plasma (1.5 kpps for 0, 5, or 30 min) The samples were diluted two-fold with PBS, mixed with chicken erythrocytes, and applied to a V-bottom plate. After incubation for 60 min, the HA titer was calculated from the weakest dilution showing hemagglutination.

was decreased to less than 1/10 for B/Johannesburg/5/99. These results were consistent with previous reports of immunochromatography regarding influenza A virus NP (3), which was degraded by the N_2 gas plasma. A hemagglutination assay demonstrated that the hemagglutination activity of the treated influenza virus was decreased from a titer of 2^9 at 0 min to a titer of 2^7 at 5 min and 2^5 at 30 min (Figure 6). These data suggest that HA was inactivated by modification and/or degradation by the N_2 gas plasma.

During N_2 gas plasma production, UV-A emission was detected by a UV-Vis-NIR spectrophotometer (data not shown). UV-A may be emitted due to the 2nd positive system of nitrogen molecules. Therefore, to investigate the effect of UV-A on the infectivity of influenza virus, allantoic fluids infected with influenza virus (A/PR/8/34) were treated with UV-A and UV-C using a UV lamp UVGL-58 for 1 and 5 min. The UVGL-58 lamp emits rays mainly at 365 nm under the UV-A generating condition (Figure 7A) and 254 nm under the UV-C generating condition (Figure 7B). After exposure of influenza A virus (A/PR/8/34) to the UV lamp, the samples were collected with pure water. Viral RNA was extracted, transcribed and subjected to rPCR using specific primers for HA, NA, M1, NS in order to investigate the effect of UV on viral RNAs. Exposure to N_2 gas plasma, UV-A, and UV-C each dramatically reduced the rPCR amplification of viral genes such as HA, NA, M1, and NS (Figure 7C). These results indicated that N_2 gas plasma, UV-A or UV-C treatment injured the genomic RNA of influenza virus. However, immunochromatography analysis of the allantoic fluid from embryonated eggs injected with the treated influenza viruses showed that virus treated with UV-A or UV-C for 1 or 5 min could still proliferate in eggs (Figure 7D), suggesting that UV-A and UV-C treatment for 5 min or less did not inactivate influenza virus in allantoic fluid. Simultaneously, these results suggest that the main mechanisms by which BLP-TESNo.1 inactivates influenza virus are not based on UV-A generated during production of the N_2 gas plasma.

The maximum temperature in the box of the N_2 gas plasma instrument during the experiment was 42°C at 5 min, 70°C at 15 min and 75°C at 30 min, as determined using a fiber thermometer (FT1420A, Takaoka, Tokyo, Japan) (Figure 8A). Because the time required to inactivate influenza virus with the N_2 gas plasma at 1.5 kpps was 5 min, the effect of heat treatment for 5 min on influenza virus was tested (Figure 8B). After heat treatment ranging from 35ºC to 65ºC for 5 min, the virus (influenza A; A/PR/8/34) was collected, injected into embryonated eggs, and incubated for 48 h to examine whether influenza virus was viable after heat treatment. We found that influenza virus retained infectivity at temperatures up to 55ºC but not those over 60ºC. These findings indicate that treatment with 45ºC, which is the temperature reached during N_2 gas plasma generation for 5 min, does not inactivate influenza virus, suggesting that heat produced during N_2 gas plasma generation does not contribute to the mechanism by which N_2 gas plasma inactivates influenza virus.

We investigated the level of oxidative stress during N_2 gas plasma generation. The color of an oxidative stress indicator (STERRAD indicator strip), which detects hydrogen peroxide-like molecules, changed after N_2 gas plasma treatment (Figure 9A). Indeed, the findings suggested that the longer the N_2 gas plasma treatment, the greater the color change of the indicator. Next, to investigate whether hydrogen peroxide-related products have disinfection activity against influenza virus, influenza A virus (A/PR/8/34) was treated with 0%–3% of hydrogen peroxide for 5 min, injected into embryonated eggs, and incubated for 48 h. The subsequent test of infectivity (Figure 9B) showed that hydrogen peroxide at more than 0.3% effectively inactivated the influenza virus (Figure 9B).

Finally, the potential oxidative changes in proteins were analyzed. Western blotting using anti-nitrotyrosine antibody showed that N_2 gas plasma treatment for 5 or 15 min increased the amount of proteins containing nitrotyrosine (Figure 10). On the basis of the molecular weight, the main viral protein stained with anti-nitrotyrosine at around 88 kDa may be nitrated NA.

5. DISCUSSION

In this study, we used an N_2 gas plasma apparatus (BLP-TESNo.1, NGK Insulators, Ltd) to inactivate influenza virus. This supports the previous study (11). After this treatment, influenza virus showed degradation and/or modification of NP, NA, HA, RNA, and lipids. The viral envelope containing lipids, HA, and NA, which are localized in the outer region of the influenza virus, was modified and/or decomposed by N_2 gas plasma. Changes in the lipids and outer surface were supported by FT-IR and SEM analysis. Furthermore, viral components localizing to the central region of the influenza particle were also degraded and/or modified by the N_2 gas plasma.

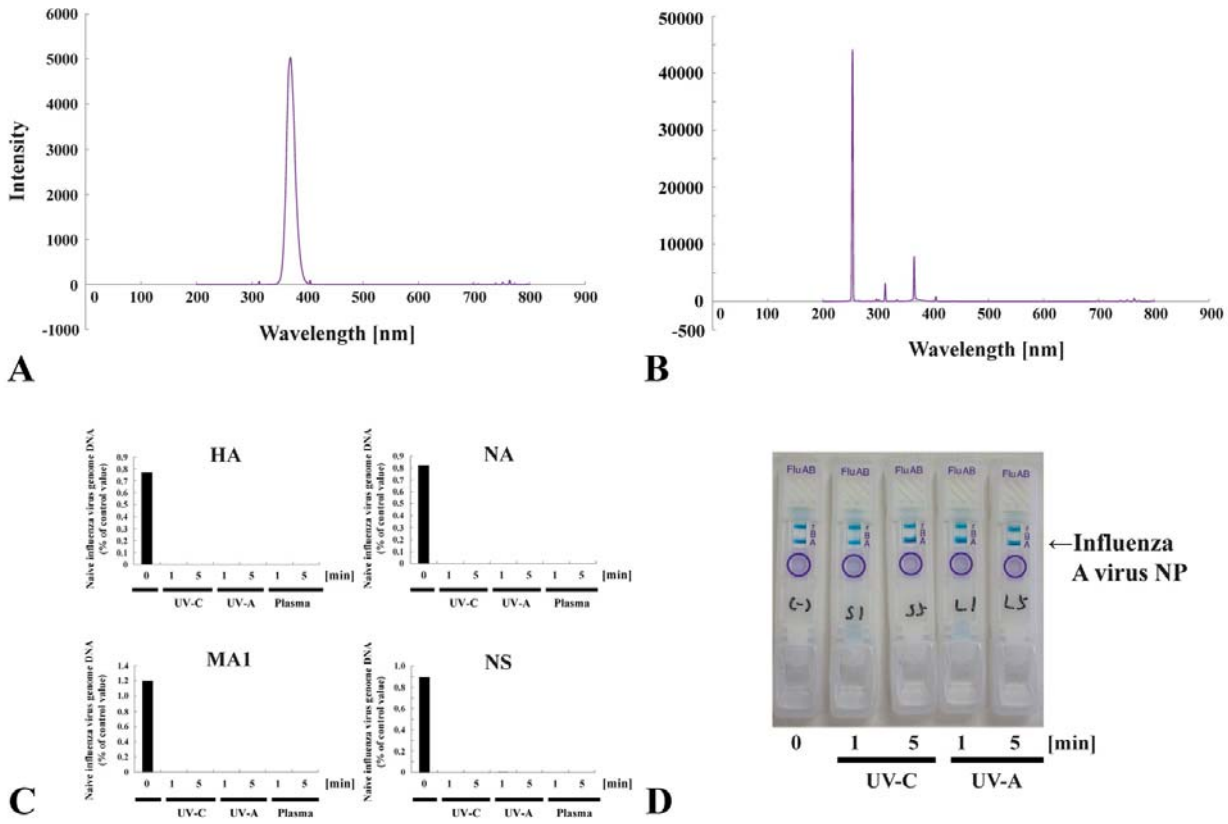

Figure 7. Treatment by UV and N_2 gas plasma causes injury to viral genomic RNA.(A,B) The UV transilluminator (UVGL-58, UVP) emits UV-A (A) and UV-C (B) around 254 nm. (C) Influenza virus (A/PR/8/34) in allantoic fluid (titer) was air-dried on a cover glass and treated with N_2 gas plasma using BLP-TESNo.1, and with UV-A and UV-C using UVGL-58 for 0, 1, or 5 min. Viral genomic RNA was extracted and subjected to real-time PCR using primers for hemagglutinin (HA), neuraminidase (NA), matrix (M1), and non-structural protein (NS) (D) Influenza virus A (A/PR/8/34; $3X10^{14}$ $TCID_{50}$/ml) treated with UV-A and UV-C using UVGL-58 for 0, 1, or 5 min was injected into embryonated eggs and incubated for 48 h. The allantoic fluids of the eggs were then collected and subjected to immunochromatography (ESPRINE Influenza A&B-N). Arrow indicates influenza A virus NP; asterisk indicates the reference line.

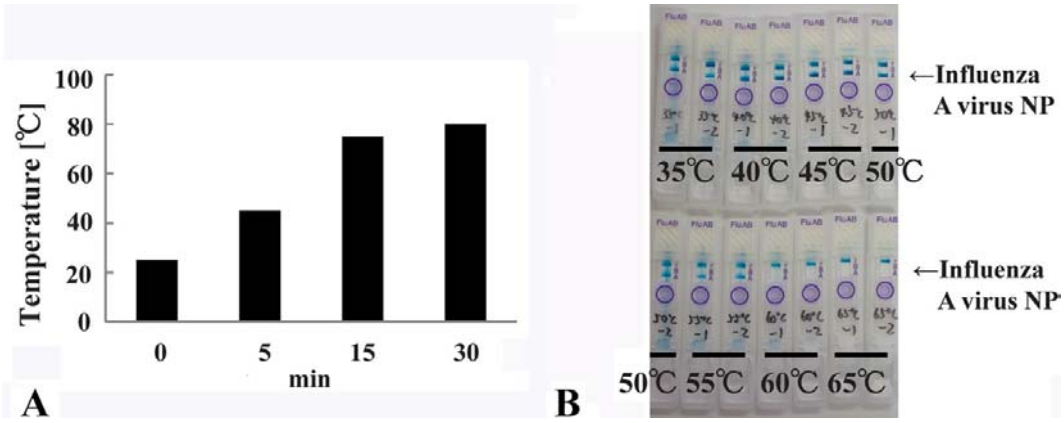

Figure 8. Effect of temperature on influenza virus during N_2 gas plasma generation. (A) The temperature in the BLP-TESNo.1 instrument during N_2 gas plasma generation was measured by a fiber optic thermometer (FT1420H, Takaoka Electric MFG. Co. Ltd., Tokyo, Japan) (B) Influenza virus (A/PR/8/34) in allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was air-dried on cover glass, treated with heat (35-65ºC; N=2) for 5 min, and then injected into embryonated eggs. Next, the eggs were incubated for 48 h, and then allantoic fluids collected from the eggs were subjected to immunochromatography (ESPRINE Influenza A&B-N) Arrow indicates influenza A virus NP; asterisk indicates the reference line.

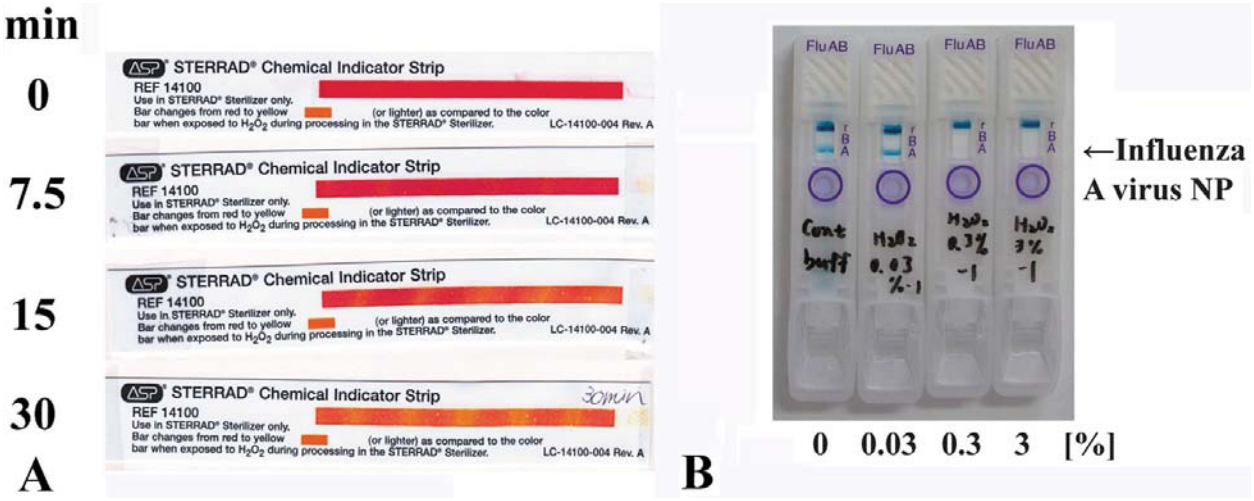

Figure 9. Treatment of influenza virus with oxidative stress and oxidative stress production during N_2 gas plasma generation.(A) Generation of hydrogen peroxide (H_2O_2)-like species during N_2 gas plasma process was detected by using a chemical indicator strip (STERRAD, Chemical Indicator Strip Ref14100). (B) Influenza virus (A/PR/8/34) in allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was treated with H_2O_2 (0-3%) for 5 min at room temperature and injected into embryonated eggs. The eggs were incubated for 48 h, and their allantoic fluids were collected and applied to immunochromatography (ESPRINE A Influenza A&B-N) Arrow indicates influenza A virus NP; asterisk indicates the reference line.

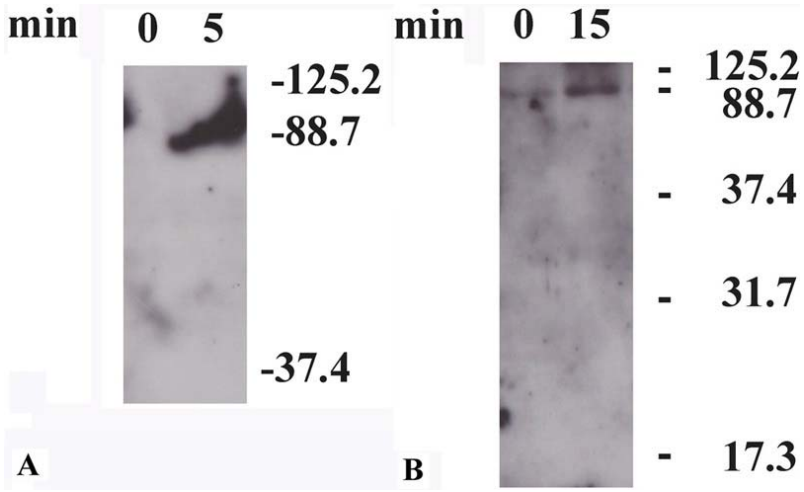

Figure 10. Nitration of proteins in influenza virus-infected allantoic fluid.Proteins in influenza virus (A/PR/8/34)-infected allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) treated with N_2 gas plasma (1.5kpps; 0, 5, 15min) were subjected to sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) and transblotted onto a polyvinylidene fluoride (PVDF) membrane. Nitrated proteins on the membrane were detected with anti-nitrotyrosine antibody. The amount of nitrated proteins was increased after N_2 gas plasma treatment. The molecular weight marker is shown on the right. The intensely stained band ~~in~~ around 88.7 kDa may be due to nitrated NA.

We believe that some of these changes may be related to the mechanisms by which the N_2 gas plasma inactivates influenza virus.

Previous studies have shown that the main inactivation factors among cells exposed to gas plasma are UV radiation, chemically reactive species, and local heat depending on the process gas used (12-15). Similarly, elevated temperature, UV emission, and oxidative stress production were also detected by, respectively, a thermometer, spectrophotometer, and chemical indicator, during N_2 gas plasma generation using BLP-TESNo.1; as a result, at least three major mechanisms of action (heat, UV, and oxidative stress) were presumed in this system. Although UV-A emission and heating (45°C, 5min) were observed, treatment by UV-A exposure or heating alone was insufficient for inactivating influenza virus. By contrast, oxidative stress (hydrogen peroxide-related products) efficiently inactivated influenza virus. In contrast to hydrogen

Table 1. Analysis of exhaust gas from BLP-TESNo.1

Gas	Concentration
NO_2	0.1-0.2ppm
NO	<10ppm
NO_x	1～3ppm
Ozone	<0.025ppm

Gas exhausted from BLP-TESNo.1 during generation of the N_2 gas plasma was analyzed by a conventional gas detector (Kitagawa Gas detector Tube System; Komyo Rikagaku Kogyo K.K., Kawasaki, Japan.

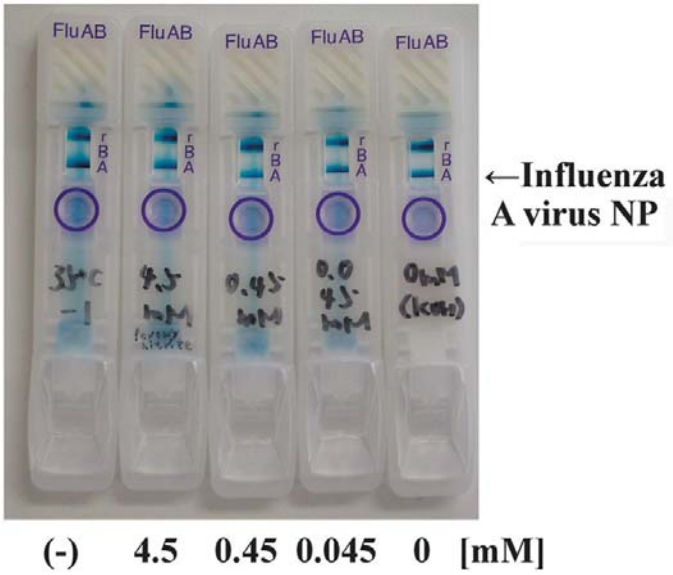

Figure 11. Treatment of influenza virus with peroxynitrite ($ONOO^-$). Influenza virus (A/PR/8/34) in allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was treated with $ONOO^-$ (0-4.5 mM) (Dojindo, Kumamoto, Japan) for 5 min at room temperature and injected into embryonated eggs. The eggs were incubated for 48 h, and their allantoic fluids were collected and applied to immunochromatography (ESPRINE A Influenza A&B-N) Arrow indicates influenza A virus NP; asterisk indicates the reference line.

peroxide, peroxynitrite and $эO_2^-$ or SIN-1 (producing $NO+эO_2^-$, resulting in peroxynitrite generation) did not influence viral activity (Figures 11-13). Therefore, oxidative stress (i.e. hydrogen peroxide-related products) may be an important factor in the inactivation of influenza virus during N_2 gas plasma production.

In relation to these results, gas analysis showed that the exhaust gas from BLP-TESNo.1 during N_2 plasma generation included NO_2 at 0.1-0.2 ppm, whereas NO, NO_x, and oxone were below the detectable limit (NO < 10 ppm, NO_x < 0.5 ppm, oxone < 0.025 ppm) (Table 1). The remaining NO_2 in the exhaust gas indicates the presence of not only N_2 but also O_2 in the atmosphere, resulting in the reaction of O_2 and N_2.

The present study suggests that oxidative stress (possibly hydrogen peroxide-related products) is the main factor in the inactivation of influenza virus during N_2 gas plasma production. The alterations in viral proteins, lipids, and RNAs induced by the N_2 gas plasma might have been due to hydrogen peroxide-like molecules. Identification of the hydrogen peroxide-like molecules that effectively inactivate influenza virus, coupled with optimization of the gas plasma generating conditions, such as the mixing of various gases, may facilitate efficient inactivation treatment. Indeed, the addition of oxygen to helium has been found to enhance the efficiency of inactivation in the case of bacteria (16). In addition, oxidation and peroxidation processes on the surface of cells and within cells result in inactivation (17, 18). Furthermore, destruction of the surface structure by gas plasma may be the main mechanism of inactivation of bacteria (19), which may be the case for viruses.

Virucidal efficiency may be also determined by the virus condition. Viruses normally interact with extracellular materials including proteins, lipids, salts, and cell debris. In addition, conditions favorable to viruses are related to resistance to virucides. Such environments include various body fluids, such as nasal fluid, blood, serum, spinal fluid, and saliva, as well as allantoic fluid. These environments prevent drying and/or stabilize the viral structure, extending the survival time of viruses. In other cases, some viruses cause the aggregation of host cells (2, 6) or make aggregates themselves (20) in a process that is called clumping, resulting in a reduction of the virucidal effect.

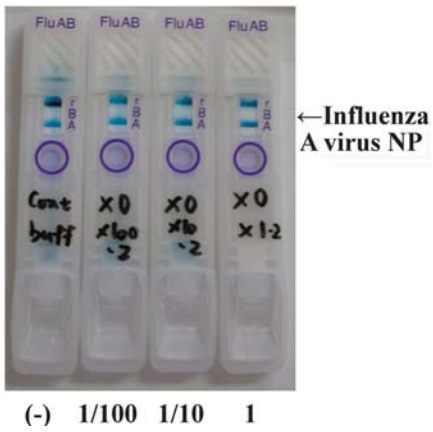

Figure 12. Treatment of influenza virus with superoxide anion ($\cdot O_2^-$). Influenza virus (A/PR/8/34) in allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was treated with $\cdot O_2^-$ (Dojindo, Kumamoto, Japan) by xanthine-oxidase system at the indicated dilutions for 5 min at room temperature and injected into embryonated eggs. The eggs were incubated for 48 h, and their allantoic fluids were collected and applied to immunochromatography (ESPRINE A Influenza A&B-N) Arrow indicates influenza A virus NP; asterisk indicates the reference line.

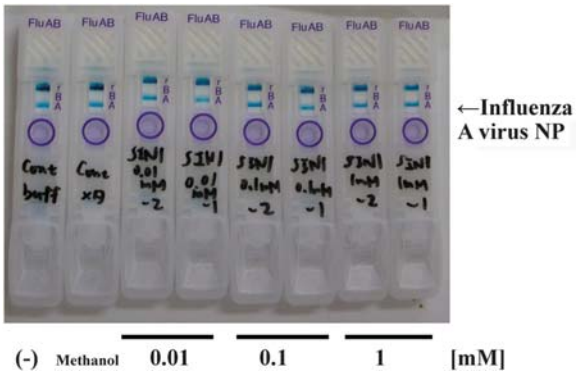

Figure 13. Treatment of influenza virus with superoxide anion ($\cdot O_2^-$). Influenza virus (A/PR/8/34) in allantoic fluid ($3X10^{14}$ $TCID_{50}$/ml) was treated with 0-1 mM of SIN-1 (Dojindo, Kumamoto, Japan) for 5 min at room temperature and injected into embryonated eggs. The eggs were incubated for 48 h, and their allantoic fluids were collected and applied to immunochromatography (ESPRINE A Influenza A&B-N) Arrow indicates influenza A virus NP; asterisk indicates the reference line.

Ultimately, we showed that an N_2 gas plasma generated by a high-voltage pulse using an SI thyrister power supply effectively inactivated influenza virus. If this method is effective against a broad range of viruses, it could contribute to infection prevention and contamination control such as sterilization of medical devices, production of safe drinking water, production and preservation of blood products, and decontamination of surfaces. In addition, it was clarified that hydrogen peroxide-like molecules produced during generation of the N_2 gas plasma are the main inactivation factor. Optimization of this main virucidal factor may increase the efficiency of this technique. The characteristics and inactivation efficiency of the gas plasma are affected by the electrical power applied, the process gas, and the gas pressure (12), and further optimization and scale-up may be necessary for the practical use of this technique. Furthermore, although the gas plasma penetrates to a depth of only about 100-1000 nm and objects can be sterilized or disinfected with limited or without damage (4, 21), the absence of injury and harmful by-products by gas plasma should be confirmed before putting this technique to practical use. In this study, we analyzed the effect of N2 gas plasma treatment on influenza virus, which has a viral envelope; however, the effectiveness of this treatment against various other pathogens, which may differ in resistance to disinfection, is unknown. For example, it would be interesting to investigate the effect of this treatment on non-enveloped viruses, such as norovirus or adenovirus, as well as protozoan oocysts and the highly resistant prion agents.

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